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I, LEANNE MYNOTT, MANAGER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PR 1614 for a patent by GRADIPORE LIMITED filed on 22 November 2000.

WITNESS my hand this
Sixteenth day of November 2001

LEANNE MYNOTT
MANAGER EXAMINATION SUPPORT AND SALES

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# AUSTRALIA

## Patents Act 1990

**Gradipore Limited**

**The Texas A&M University System**

**PROVISIONAL SPECIFICATION**

*Invention Title:*

*Apparatus and method for isoelectric focusing separations*

The invention is described in the following statement:

Technical Field

The present invention relates to a principle, method and apparatus to carry out analytical and preparative-scale isoelectric focusing (IEF) separations.

Background Art

Separation of amphoteric molecules according to their isoelectric points (pI) by isoelectric focusing (IEF) has proven to be a highly successful and popular technique. The resolution offered by analytical IEF is amongst the highest available from present biochemical separation techniques. Current methods for preparative IEF, however, generally do not meet the need of scale and speed of separation, low cost, and ease of use.

The closest known methods are the isoelectric focusing separations in ampholytes or binary buffers (Bier-buffers) in the Rotophor unit or in the recirculating preparative electrophoretic systems (e.g., in the RF-3 unit, marketed by Protein Technologies, Tucson, AZ, USA) or in the free-flow electrophoretic systems (e.g., in the Octopus unit, marketed by Weber GmbH, Kirchheim-Heimstetten, Germany), as well as the isoelectric focusing separations in multi-compartment, isoelectric membrane electrolyzers (e.g., in the IsoPrime unit, marketed by Amersham-Pharmacia, San Franciso, CA, USA).

In order to carry out an IEF separation, a pH gradient has to be established. A pH gradient can be created in ampholyte mixtures or in binary weak electrolyte mixtures (such as Bier's buffers). The pH gradients can be stabilized with the help of either anti-convective media (such as gels or capillaries) or by well-controlled laminar flows (free-flow electrophoretic systems). The drawback of both of these approaches is that the separated components also contain the components of the media used to establish the pH gradient. A significant improvement was offered by Righetti's invention (described in US Patent Numbers 4,971,670 and 5,082,548), the multi-compartment isoelectric membrane electrolyzer (IsoPrime). Righetti realized that an IEF separation can be accomplished by creating a series of isoelectric membranes such that the membrane pI values fall in-between the pI values of the sample components, and placing these membranes between an anodic (low pI) isoelectric membrane and a cathodic (high pI) isoelectric membrane to provide interfaces permeable to hydronium and hydroxyl ions. Under the

influence of the electric field, the sample components are trapped between the membranes whose pI values bracket the pI value of the sample component. Thus, an IEF separation in the IsoPrime unit does not require the presence of an electrolyte in addition to the sample component.

The crucial features of Righetti's system call for the use of either immobilized pH gradient gels or isoelectric membranes for the isolation of the anode compartment, the separation chamber and the cathode compartment.

Gradiflow™ is a technology originally developed for the separation of macromolecules such as proteins, nucleotides and complex sugars. The Gradiflow™ process provides a scalable separation that is faster, cheaper and yields larger amounts of high-purity products than current methods of macromolecule separation and offers the potential to concurrently purify and detoxify or decontaminate macromolecule solutions.

The Gradiflow™ technology utilises tangential flow along a membrane when an electric field or potential is applied across the membrane (AU 601040). Some examples of Gradiflow™ technology may be found in US Patent Numbers 5039386 and 5650055, which US Patents are incorporated herein by reference.

In essence, the Gradiflow™ technology (Gradipore Limited, Australia) is bundled into a cartridge comprising at least three membranes housed in a system which allows separation of macromolecules by charge and/or molecular weight. The system can also concentrate and desalt/dialyse at the same time. The multimodal nature of the system allows this technology to be used in a number of other areas especially in the production of biological components for medical use. The structure of the membranes may be configured so that biological contaminants can also be removed at the point of separation – a task which is not currently available in the biotechnology industry and which adds to the cost of production through time delays and due to the complexity of the task.

An IEF system has now been devised by the present inventors by adapting the Gradiflow™ system. The system is based on the finding that a functional equivalent (for IEF purposes) of an amphoteric isoelectric membrane can be created from a solution of an amphoteric isoelectric substance and at least two interfaces that permit passage of ions but substantially prevent pressure- or gravity-driven hydraulic flow. The solution of the amphoteric isoelectric substance can be stationary, flowing or

recirculated between the ion-permeable interfaces that prevent pressure- or gravity driven hydraulic flow.

Disclosure of Invention

The present invention relates to a principle, method and apparatus to carry out analytical and preparative-scale isoelectric focusing (IEF) separations. The application areas of the new principle, method and apparatus are in the separation, purification, enrichment, concentration or conditioning of both small and large molecular weight ampholytic compounds, such as small ampholytic pharmaceuticals (natural and non-natural amino acids, aminophenolics, amino phosphonic acids), oligo- and polypeptides, proteins, and other biomolecules. These separations can be achieved based on the use of protic equilibria only, or by a combination of protic and other (e.g., complexation) secondary chemical equilibria. Although such operations could be achieved by other means, such as via the use of ampholytes, immobilized pH-gradient gels or isoelectric membranes, the method according to the present invention offers greater simplicity and possibly higher production/separation rates.

In a first aspect, the present invention provides an electrophoresis system for isoelectric focusing of a compound, the system comprising:-

(a) an anode positioned in an anode-compartment containing an anolyte;

(b) an anodic gateway, adjacent to the anode-compartment, containing an isoelectric solution or liquid and comprising at least one ion-permeable interface or barrier, such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized;

(c) a cathode positioned in a cathode-compartment containing a catholyte;

(d) a cathodic gateway, adjacent to the cathode-compartment, containing an isoelectric solution or liquid and comprising at least one ion-permeable interface or barrier, such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; and

(e) an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one separation compartment.

In a preferred embodiment of the first aspect, the present invention provides an electrophoresis system for isoelectric focusing of a compound, the system comprising:-

(a) an anode positioned in an anode-compartment containing an anolyte;

(b) an anodic gateway, adjacent to the anode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized;

(c) a cathode positioned in a cathode-compartment containing a catholyte;

(d) a cathodic gateway, adjacent to the cathode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; and

(e) an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one upper and at least one lower separation compartment.

In another embodiment of the first aspect, the present invention provides an electrophoresis system for isoelectric focusing of a compound, the system comprising:-

(a) an anode positioned in an anode-compartment containing an anolyte;

(b) an isoelectric membrane, adjacent to the anode-compartment, such that convective mixing between the anodic solution and any liquid in an adjacent compartment is minimized;

(c) a cathode positioned in a cathode-compartment containing a catholyte;

(d) a cathodic gateway, adjacent to the cathode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; and

(e) an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one upper and at least one lower separation compartment.

In yet another embodiment of the first aspect, the present invention provides an electrophoresis system for isoelectric focusing of a compound, the system comprising:-

(a) an anode positioned in an anode-compartment containing an anolyte;

(b) an anodic gateway, adjacent to the anode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized;

(c) a cathode positioned in a cathode-compartment containing a catholyte;

(d) an isoelectric membrane, adjacent to the cathode-compartment, such that convective mixing between the cathodic solution and any liquid in an adjacent compartment is minimized; and

(e) an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one upper and at least one lower separation compartment.

In another preferred implementation of any of the above embodiments, the system has several isoelectric partitioners positioned between the anode- and cathode-compartments forming multiple upper and lower separation compartments on either side of the partitioners.

In yet another preferred implementation of any of the above embodiments, the system has only one separation compartment formed between the anode and cathode compartments.

In a still further embodiment of the first aspect of the present invention, the system further includes:-

(e) means for circulating at least one of the materials in the upper and lower separation compartments, solutions in the anode- and cathode-compartments, solutions in the anodic or cathodic gateways, and solutions in the isoelectric partitioners, if used.

In an other embodiment of the first aspect of the present invention, the system further includes:-

(f) means for removing and replacing solutions and/or sample(s) in any or all of the compartments.

An example of a suitable material for the anodic isoelectric gateway is iminodiacetic acid. An example of a suitable material for the cathodic isoelectric gateway is lysine. It will be appreciated, however, that other materials would also be suitable.

The isoelectric partitioner can be an isoelectric membrane or, preferably, a functional equivalent of an isoelectric membrane. The functional equivalent of an isoelectric membrane can be created by enclosing, between two ion-permeable interfaces, a solution of an isoelectric substance that offers good buffering capacity and adequate conductivity around its pI value. An ion-permeable interface can be a membrane (non-ionic, ionic or isoelectric), an immiscible liquid or a porous solid. The isoelectric substances enclosed between the ion-permeable interfaces can be molecules with appropriate combinations of weak acid and weak base functionalities,

weak acid and strong base functionalities, or strong acid and weak base functionalities. For example, suitable isoelectric substances can be (poly)amino (poly)carboxylic acids, (poly)amino (poly)phenols, (poly)amino (poly)phosphonic acids, (poly)amino (poly)sulfonic acids, (poly)amino (poly)phenol(poly)carboxylic acids, (poly)amino (poly)-phenol(poly)phosphonic acids, (poly)amino (poly)carboxylic (poly)-phosphonic acids, (poly)amino (poly)phenol(poly)sulfonic acids, (poly)amino (poly)phenol- (poly)carboxylic(poly)sulfonic acids or (poly)amino (poly)phenol(poly)carboxylic- (poly)phosphonic(poly)sulfonic acids or their combinations. Theoretically, any desired pI can be established for a given application by providing an isoelectric solution that offers good buffering capacity and adequate conductivity around its pI value in the isoelectric partitioner.

The ion-permeable interface or barrier can be formed of any suitable material. In one preferred form, the ion-permeable interface or barrier is made from polyacrylamide. If desired, the ion-permeable interface can restrict the passage of certain molecules greater than a specified size. Preferably, the ion-permeable, convective mixing-preventing interface is a non-ionic membrane (unsupported, such as cellulose acetate, cross-linked polymethacrylate, or supported such as cross-linked polyacrylamide, agar, etc., on glass fiber filter or PET paper), or a porous frit (e.g., glass frit, polymeric frit, etc.). Two such interfaces are used to enclose the stagnant, flowing or recirculated solution of the isoelectric material that has sufficient conductivity and buffering/titrating capacity.

In the embodiment of the system where the anodic and/or the cathodic gateway has only one ion-permeable membrane, the isoelectric solution in the gateway also forms the anolyte and/or catholyte in the respective anode- and cathode-compartments. The ion-permeable membrane is thus positioned adjacent to the isoelectric solution thereby minimizing mixing with any liquid in the adjacent compartment.

The temperature of buffers, isoelectric solutions and sample solutions in the system can be controlled by any suitable cooling/heating means. The system may also be positioned in a controlled-temperature environment to maintain a desired temperature during operation.

The atmosphere in contact with any or all of the buffers, isoelectric solutions and sample solutions in the system can be controlled by any

suitable gas handling system. The system may also be positioned in a controlled chemical composition environment to maintain a desired atmosphere during operation.

The system may have its own power supply or can be connected to an external power supply.

In one preferred form, part of the system which contains the isoelectric gateway, isoelectric membrane, and isoelectric partitioner forming the upper and lower separation compartments is provided as a cartridge or cassette adapted to be positioned between the anode and cathode.

The distance between the electrodes (anode and cathode) can have an effect on the separation or movement of compounds through the various barriers or interfaces. As the electric field strength has an important effect on the separation, shorter distances between the electrodes are often advantageous.

The ion-permeable interface or barrier and the isoelectric partitioner may be formed as a multilayer or sandwich arrangement. As the electric field strength has an important effect on the separation, the thickness of the materials can have an effect on the separation of the sample components. It has been found in many circumstances that thinner elements are often advantageous.

In the embodiments where the sample and/or isoelectric solutions are not stagnant, flow rates of the electrolyte and/or sample solutions through the system can have an influence on the temperature profile in the system and thus, can have an effect on the separation of the sample components.

Field strengths across the system can vary depending on the separation. Typically, field strength can be up to 1000 V/cm, depending on the configuration of the system, and the composition of the electrolyte and sample solutions used.

In use, a sample to be treated is placed in, or recirculated through, at least one separation compartment, any or all of which may contain suitable additional electrolytes or component(s); appropriate solutions placed in, or recirculated through, the anode- and cathode-compartments; appropriate solutions placed in, or recirculated through, the anodic isoelectric gateway, if used, and cathodic isoelectric gateway, if used, or both, if used; an appropriate anodic isoelectric membrane placed, if used, adjacent to the anode compartment; an appropriate cathodic isoelectric membrane placed, if

used, adjacent to the cathode compartment; the isoelectric partitioner(s) is (are) selected, if used, according to the desired pI value(s) as described above; an electric potential is applied to the system via the anode and cathode thereby causing one or more compounds in the sample to move through the isoelectric partitioner(s) and/or isoelectric gateway(s) for disposal, collection or further treatment.

It will be appreciated that the embodiments outlined above can be implemented in simplified arrangements without sacrificing the objectives of this invention. For example, in one such embodiment, the essential functions of the anode-compartment and the anodic isoelectric gateway can be merged by using an isoelectric solution as the anolyte and a single ion-permeable barrier that minimizes convective mixing between the anolyte and the adjacent separation compartment.

In another such embodiment, the essential functions of the cathode-compartment and the cathodic isoelectric gateway can be merged by using an isoelectric solution as the catholyte and a single ion-permeable barrier that minimizes convective mixing between the catholyte and the adjacent separation compartment.

In yet another such embodiment, the essential functions of both the anode compartment and the anodic isoelectric gateway and the cathode compartment and the cathodic isoelectric gateway can be merged by using one isoelectric solution as the anolyte and a single ion-permeable barrier that minimizes convective mixing between the anolyte and the adjacent separation compartment and another isoelectric solution as the catholyte and a single ion-permeable barrier that minimizes convective mixing between the catholyte and the adjacent separation compartment.

In a second aspect, the present invention provides a method for separating at least one component from a sample by isoelectric focusing, the method comprising the steps of:-

(a) placing the sample in a separation compartment of an electrophoresis system comprising an anode positioned in an anode-compartment containing an anolyte; an anodic gateway, adjacent to the anode-compartment, containing an isoelectric solution or liquid and comprising at least one ion-permeable interface or barrier, such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; a cathode positioned in a cathode-compartment containing a catholyte; a

cathodic gateway, adjacent to the cathode-compartment, containing an isoelectric solution or liquid and comprising at least one ion-permeable interface or barrier, such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; and an isoelectric partitioner positioned between the anode- and cathode-compartments forming the separation compartment;

(b) providing suitable electrolytes or solutions to the anode- and cathode-compartments, the anodic and cathodic gateways and the separation compartment;

(c) selecting the isoelectric partitioner according to the desired pI value; and

(d) applying an electric potential between the anode and cathode causing at least one compound from the sample to migrate.

In a preferred embodiment of the second aspect, the present invention provides a method for separating at least one component from a sample by isoelectric focusing, the method comprising the steps of:-

(a) placing the sample in at least one of the upper or lower separation compartments of an electrophoresis system comprising an anode positioned in an anode-compartment containing an anolyte; an anodic gateway, adjacent to the anode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; a cathode positioned in a cathode-compartment containing a catholyte; a cathodic gateway, adjacent to the cathode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; an isoelectric partitioner positioned between the anode and cathode compartments forming at least one upper and at least one lower separation compartments;

(b) providing suitable electrolytes or solutions to the anode- and cathode-compartments, the anodic and cathodic gateways and the upper and lower separation compartments;

(c) selecting the isoelectric partitioner according to the desired pI value; and

(d) applying an electric potential between the anode and cathode causing at least one compound from the sample to migrate.

In a further preferred embodiment of the second aspect, the present invention provides a method for separating at least one component from a sample by isoelectric focusing wherein step (a) comprises:-

placing the sample in at least one of the upper or lower separation compartments of an electrophoresis system comprising an anode positioned in an anode-compartment containing an anolyte; an isoelectric membrane, adjacent to the anode-compartment, such that convective mixing between the anodic solution and any liquid in an adjacent compartment is minimized; a cathode positioned in a cathode-compartment containing a catholyte; a cathodic gateway, adjacent to the cathode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; and an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one upper and at least one lower separation compartment.

In a further preferred embodiment of the second aspect, the present invention provides a method for separating at least one component from a sample by isoelectric focusing wherein step (a) comprises:-

placing the sample in at least one of the upper or lower separation compartments of an electrophoresis system comprising an anode positioned in an anode-compartment containing an anolyte; an anodic gateway, adjacent to the anode-compartment, containing, between two ion-permeable interfaces or barriers, an isoelectric solution or liquid such that convective mixing of the isoelectric solution or liquid and any liquid in an adjacent compartment is minimized; a cathode positioned in a cathode-compartment containing a catholyte; an isoelectric membrane, adjacent to the cathode-compartment, such that convective mixing between the cathodic solution and any liquid in an adjacent compartment is minimized; and an isoelectric partitioner positioned between the anode- and cathode-compartments forming at least one upper and at least one lower separation compartment.

The method according to the second aspect of the present invention can be used to separate any amphoteric compound including small molecules,, chiral molecules, macromolecules including proteins, peptides, nucleic acids. The method can also be used to desalt solutions containing compounds of interest.

In a third aspect, the present invention provides use of the system according to the first aspect of the present invention in the separation of at least one compound from a sample by isoelectric focusing.

In a fourth aspect, the present invention provides a compound separated by the method according to the second aspect of the present invention.

In a fifth aspect, the present invention provides a functional equivalent of an isoelectric membrane for use in an electrophoresis system for isoelectric focusing comprising enclosing, between two ion-permeable interfaces, a solution of an isoelectric substance that offers good buffering capacity and adequate conductivity around its pI value.

The ion-permeable interface can be a membrane (non-ionic, ionic or isoelectric), an immiscible liquid or a porous solid.

The ion-permeable interface can also be a membrane that has a defined molecular mass cut-off so as to only allow movement of compounds smaller that the membrane cut-off.

The isoelectric substances enclosed between the ion-permeable interfaces can be molecules with appropriate combinations of weak acid and weak base functionalities, weak acid and strong base functionalities, or strong acid and weak base functionalities. For example, suitable isoelectric substances can be (poly)amino (poly)carboxylic acids, (poly)amino (poly)phenols, (poly)amino (poly)phosphonic acids, (poly)amino (poly)sulfonic acids, (poly)amino (poly)phenol(poly)carboxylic acids, (poly)amino (poly)- phenol(poly)phosphonic acids, (poly)amino (poly)carboxylic (poly)- phosphonic acids, (poly)amino (poly)phenol(poly)sulfonic acids, (poly)amino (poly)phenol-(poly)carboxylic(poly)sulfonic acids or (poly)amino (poly)phenol(poly)carboxylic- (poly)phosphonic(poly)sulfonic acids or their combinations.

In a sixth aspect, the present invention provides a cartridge for use in a electrophoresis system for isoelectric focusing according to the first aspect of the present invention, the cartridge comprising:

(a) a housing containing an anodic isoelectric gateway, and/or a cathodic isoelectric gateway;

(b) an anodic isoelectric membrane or a cathodic isoelectric membrane, if used;

(c) an isoelectric partitioner positioned between the anodic and cathodic isoelectric gateway or the membrane(s); and

(d) one or more separation compartments on at least one side of the isoelectric partitioner, the partitioner being formed between the isoelectric gateway(s) or the isoelectric membrane(s) and the isoelectric partitioner(s), , or between different isoelectric partitioners.

Preferably, the isoelectric partitioner is formed by enclosing, between two ion-permeable interfaces, a solution of an isoelectric substance that offers good buffering capacity and adequate conductivity around its pI value. In use, the solution is preferably passed or circulated between the ion-permeable interfaces.

The ion-permeable interface can be a membrane (non-ionic, ionic or isoelectric), an immiscible liquid or a porous solid.

The isoelectric substances enclosed between the ion-permeable interfaces can be molecules with appropriate combinations of weak acid and weak base functionalities, weak acid and strong base functionalities, or strong acid and weak base functionalities. For example, suitable isoelectric substances can be (poly)amino (poly)carboxylic acids, (poly)amino (poly)phenols, (poly)amino (poly)phosphonic acids, (poly)amino (poly)sulfonic acids, (poly)amino (poly)phenol(poly)carboxylic acids, (poly)amino (poly)- phenol(poly)phosphonic acids, (poly)amino (poly)carboxylic (poly)- phosphonic acids, (poly)amino (poly)phenol(poly)sulfonic acids, (poly)amino (poly)phenol-(poly)carboxylic(poly)sulfonic acids or (poly)amino (poly)phenol(poly)carboxylic- (poly)phosphonic(poly)sulfonic acids or their combinations.

The cartridge may be disposable or adapted for multiple use, preferably with cleaning in-between uses.

Gradiflow™ is a trade mark owned by Gradipore Limited, Australia.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any description of prior art documents herein is not an admission that the documents form part of the common general knowledge of the relevant art in Australia.

In order that the present invention may be more clearly understood preferred forms will be described with reference to the following examples and drawings.

Brief Description of Drawings

Figure 1 shows a schematic representation of one embodiment of the system according to the present invention where the respective anode- and cathode-compartments are separated from the separation compartments by the anodic and cathodic isoelectric gateways, respectively, and the separation compartments are formed between the anodic gateway and the isoelectric membrane and the isoelectric membrane and the cathodic gateway.

Figure 2 shows a schematic representation of another embodiment of the system according to the present invention where the respective anode- and cathode-compartments are separated from the separation compartments by the anodic and cathodic isoelectric gateways, respectively, and the separation compartments are formed between the anodic gateway and the isoelectric separator and the isoelectric separator and the cathodic gateway.

Figure 3 shows a schematic representation of yet another embodiment of the system according to the present invention wherein the anode is in an isoelectric anolyte solution and the cathode is in an isoelectric catholyte solution, and the anode and cathode compartments are separated from the adjacent separation compartments by ion-permeable barriers that minimize convective mixing between the anolyte and the catholyte and the adjacent separation chambers.

Modes for Carrying Out the Invention

The system according to the present invention is adaptable for the separation of many different compounds having closely spaced pI values over a wide range of pI values. The system is scalable so that commercial separation of compounds can be achieved.

The functional equivalent of an isoelectric focusing apparatus can be created as shown in Figures 2 and 3 by connecting, in series, an anode, an anode-compartment, an anodic isoelectric gateway with an effective pI value

of $pI_{anodic\ gateway}$, a separation compartment, a cathodic isoelectric gateway with an effective pI value of $pI_{cathodic\ gateway}$, a cathode-compartment and a cathode. The mixture of ampholytic compound(s) to be processed or separated (sample solution) is placed into the separation chamber, between the anodic and cathodic isoelectric gateways. As usual in IEF, the anolyte might be an acidic solution or an amphiprotic substance solution with a $pI_{anolyte} < pI_{anodic\ gateway}$, the catholyte might be a base solution or an amphiprotic substance solution with a $pI_{cathodic\ gateway} < pI_{catholyte}$. As described previously, either the anodic or the cathodic isoelectric gateways can be substituted with a corresponding isoelectric membrane (Figure 3). Any or all of the solutions (anolyte, catholyte, anodic isoelectric gateway solution, cathodic isoelectric gateway solution and sample solution) might be stationary, move through the apparatus in a single pass, move through the apparatus in multiple passes or be recirculated through the apparatus during all or part of the processing steps (IEF).

The IEF separation of the sample components is achieved by placing at least one isoelectric partitioner or isoelectric membrane into the separation chamber, such that the pI value(s) of the isoelectric partitioner(s) or isoelectric membrane(s) fall between those of the anodic and the cathodic isoelectric gateways such that $pI_{anodic\ isoelectric\ gateway} < pI_{isoelectric\ partitioner} < pI_{cathodic\ isoelectric\ gateway}$. Alternatively, a series of ion-permeable membranes can be used such that $pI_{anodic\ isoelectric\ gateway} < pI_{isoelectric\ membrane} < pI_{cathodic\ isoelectric\ gateway}$. In yet another alternative, the separation compartment can be subdivided by ion-permeable membranes allowing the division of the sample into two or more fractions with different effective pI values. These fractions can be further fractionated or processed, on-line or off-line, continuously or intermittently, to create further fractions with higher purity, concentration, different composition or different effective pI values.

The quality of the IEF separation of the sample components might be further improved by adding to the sample mixture one or more ampholytic additives such that the condition $pI_{anodic\ isoelectric\ gateway} < pI_{additive} < pI_{cathodic\ isoelectric\ gateway}$ is fulfilled for each additive.

The quality of the IEF separation of the sample components might be further improved by simultaneously involving, in addition to the protic equilibria, one or more of the sample constituents in additional secondary chemical equilibria, such as complexation, association, affinity interactions,

partitioning, adsorption, evaporation, precipitation or reaction steps, on-line or off-line, continuously or intermittently, to create fractions with higher purity, concentration, different composition or different effective pI values.

The quality of the IEF separation of the sample components might be further improved by simultaneously involving, in addition to the protic equilibria and/or additional secondary chemical equilibria, one or more of the sample constituents in additional size or mobility-dependent separation steps, on-line or off-line, continuously or intermittently, to create fractions with higher purity, concentration, different composition or different effective pI values.

An IEF system has been devised by adapting the Gradiflow™ system (Gradipore Ltd, Australia). The system is based on the finding that a functional equivalent (for IEF purposes) of an immobilized pH gradient gel or amphoteric isoelectric membrane can be created from a solution of an amphoteric isoelectric substance and two interfaces that permit passage of ions but substantially prevent convective mixing between the adjacent compartments.

Typically, the ion-permeable barrier that prevents convective mixing can be a non-ionic membrane (unsupported, such as cellulose acetate, cross-linked polymethacrylate, or supported such as cross-linked polyacrylamide, agar, etc., on glass fiber filter or PET paper), or a porous frit (e.g., glass frit, polymeric frit, etc.). Two such barriers can be used to enclose the stagnant, flowing or recirculated solution of an amphiprotic, isoelectric material that has suitable conductivity and buffering capacity and is used either in the anodic isoelectric gateway, and/or the cathodic isoelectric gateway, and/or the isoelectric partitioner.

The isoelectric partitioner (located in the separation chamber) can be an isoelectric membrane whose pI value can be selected, or an isoelectric partitioner containing the isoelectric medium whose pI value can also be selected. A typical medium would be isoelectric polymeric buffers synthesized from acidic and basic acrylamido monomers and acrylamide, without cross-linkers.

A single such isoelectric partitioner can lead to a binary separation, i.e., the sample is divided into two fractions: one of the fractions has a lower, the other one a higher pI value than the isoelectric partitioner. Narrow pI cuts can be obtained by two sequential IEF separations or by two sequentially

connected isoelectric chambers with different isoelectric partitioner pI values. Alternatively, multiple (n) isoelectric partitioners could be used in a single separation chamber to produce (n-1) fractions.

Another embodiment of the present invention relates to the use of a large number of non-isoelectric, but ion-permeable membrane barriers (e.g., cellulose acetate or cross-linked acrylamide membranes) to divide the separation chamber into multiple compartments. A mixture of amphiprotic compounds would then focus across the separation chamber and accumulate in different chambers. The greater the number of membrane barriers, the finer the pI resolution for the bands.

A simpler, alternative embodiment is directly applicable to the a cartridge system as follows. The anode compartment is filled with the solution of a low pI isoelectric amphiprotic substance. The cathode compartment is filled with the solution of a high pI substance. The anode and cathode compartments are interfaced to the separation chamber (upstream and downstream channels) by two non-ionic membranes. The upstream and downstream channels are separated by an isoelectric membrane whose pI value will determine the cut-point of the IEF separation. By repeating the separation, using isoelectric membranes of closely spaced pI values, narrow pI cuts of compounds could be produced.

By implementing the IEF membrane functional equivalent in a size-exclusion membrane matrix, simultaneous size-based and pI-based separations could be obtained.

By using additives, such as cyclodextrins, simultaneous secondary chemical equilibria can be implemented on top of the protic equilibria. This would lead to improved separations and/or new kinds of separations, such as enantiomer or positional isomer separations.

By using this invention, fast separations can be obtained with relatively low applied potential, because the distance between the electrode compartments is minimal. The surface area of the membranes can be easily increased to increase production rate.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty-second day of November 2000

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Patent Attorneys for the Applicants:

~~F B RICE & CO~~
ALLENS

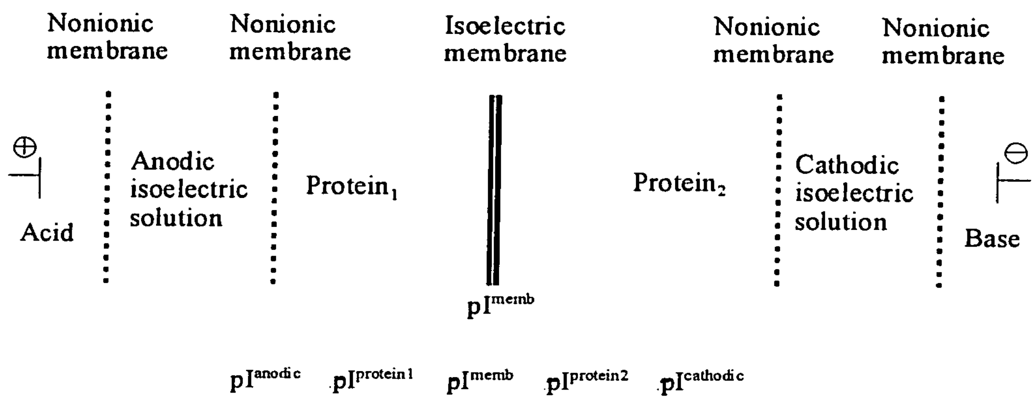
Nonionic membrane
Nonionic membrane
Isoelectric membrane
Nonionic membrane
Nonionic membrane
Acid
Anodic isoelectric solution
Protein₁
Protein₂
Cathodic isoelectric solution
Base
pI^memb
pI^anodic pI^protein1 pI^memb pI^protein2 pI^cathodic

Figure 1

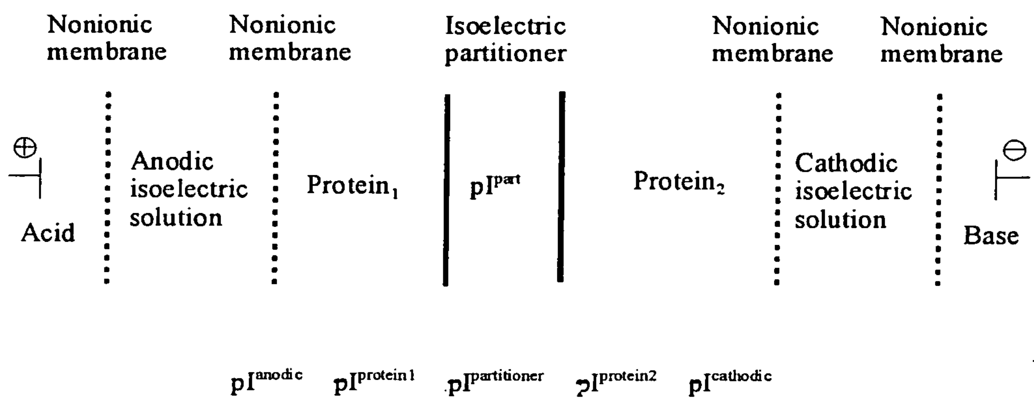
Nonionic membrane
Nonionic membrane
Isoelectric partitioner
Nonionic membrane
Nonionic membrane
⊕
Acid
Anodic isoelectric solution
$Protein_1$
$pI^{part}$
$Protein_2$
Cathodic isoelectric solution
⊖
Base
$pI^{anodic}$
$pI^{protein1}$
$pI^{partitioner}$
$pI^{protein2}$
$pI^{cathodic}$

Figure 2

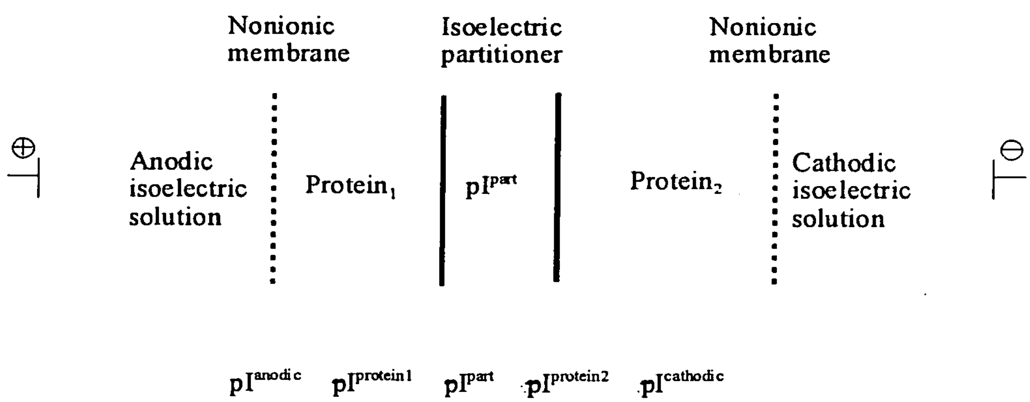
Nonionic membrane
Isoelectric partitioner
Nonionic membrane
⊕
Anodic isoelectric solution
Protein1
pIpart
Protein2
Cathodic isoelectric solution
⊖
pIanodic
pIprotein1
pIpart
pIprotein2
pIcathodic

Figure 3